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(54) Title: OSTEOPONTIN COATED SURFACES AND METHODS OF USE

(57) Abstract

A novel osteopontin containing implant which increases the rate of osseointegration and the percentage of bone apposition is described. The implant of the invention includes a material suitable for use *in vivo* within a subject in combination with a releasable form of osteopontin forming an osteopontin containing implant.

OSTEOPONTIN COATED SURFACES AND METHODS OF USE

Background of the Invention

The process that leads to successful osseointegration of an implant into the surrounding tissues is a complex one that involves cell migration, attachment, differentiation, proliferation, extracellular matrix synthesis and finally mineralization of that matrix. Implant materials are as biocompatible as their surface chemistry allows for a favorable interaction with the biological molecules relevant for that tissue.

For example, placement of endosseous dental implants has been limited to areas of favorable bone character, and fixtures must remain unloaded after placement for considerable periods of time. The primary challenges faced in the fabrication of new endosseous implants are to increase the rate of osseointegration and the percentage of bone apposition. Histological analysis of integrated titanium (Ti) implants into bone tissue revealed that many clinically successful implants are among 30 - 60 % opposed directly by mineralized bone. The rest of the implant surface has been found to be apposed by fibrous tissue and unmineralized collagen fibers. It is desirable that the entire circumference of the osseointegrated implant be directly apposed by mineralized bone tissue.

Extracellular matrix proteins, especially certain adhesion molecules, play a role in bone repair and morphogenesis. These molecules can modulate gene expression through cell surface-extracellular matrix interactions. The interaction between the titanium oxide layer of dental implants and certain extracellular matrix proteins may be a prerequisite for reproducible direct apposition of bone to titanium implants.

Human osteoblast cell lines undergo a coordinated temporal expression of osteoblast phenotypic markers during their differentiation *in vitro* and produce a mineralized extracellular matrix. This bone developmental system is ideal for studying the interaction between titanium surfaces and bone cells *in vitro*.

Summary of the Invention

The implants of the invention are improved implants which increase the rate of osseointegration and the percentage of bone apposition. Implant surfaces should have such properties which permit the phenomenology of the relevant cells. The achievement of reproducible biological integration of implants calls for a delineation of the molecular biological events relevant to the morphogenesis of the desired interfacial tissue. Material surfaces that can not bind the macromolecules supportive of osteoblast function, are not likely to make a good bone implant.

In another aspect the invention features a method of inducing new bone formation in a subject at a site where bone formation is needed. The method includes implanting an osteopontin containing implant into a subject at a site where bone formation is needed, wherein the osteopontin is released from the implant into the site thereby inducing new bone formation at the site.

In another aspect the invention features an osteopontin containing cell recruitment system. The system includes a releasable osteopontin or a fragment thereof in a form which provides a gradient and an implant, forming a cell recruitment system in the proximity of the implant, wherein the implant is targeted for cell recruitment by a gradient of osteopontin which forms in the proximity of the implant.

In another aspect the invention feature a packaged releasable osteopontin or a fragment thereof for use in a cell recruitment system. The package includes a releasable osteopontin or a fragment thereof in a form which provides a gradient in the proximity of an implant which is targeted for cell recruitment by the gradient of osteopontin, packaged with instructions for use of said osteopontin or a fragment thereof with the implant targeted for cell recruitment.

In another aspect the invention features a coated osseointegrator capable of implantation. The osseointegrator includes a coated material which is enhanced for ooseointegration by at least about 100% when compared to an uncoated material based on the human osteoblast cell (HOS) attachment assay.

In another aspect the invention features a coated implant. The implant includes a coated material which increases the proliferation of osteoblasts by at least about 100% when compared to an uncoated material based on the human osteoblast cell (HOS) proliferation assay.

In still another aspect, the invention features a method for inducing new tissue formation in a subject at a site where tissue formation is needed. The method includes adding osteopontin into a subject at a site where tissue formation is needed, wherein the osteopontin induces new tissue formation about the site.

In yet another aspect, the invention features an osteopontin glue which includes osteopontin, a mucopolysaccharide and a multivalent metal, e.g., calcium, magnesium or manganese. Preferably, the osteopontin is at a concentration of about 100 µg/g of glue.

osteopontin phosphorylated at one or more of the following amino acids selected from the group consisting of Ser26, Ser27, Ser63, Ser76, Ser78, Ser81, Ser99, Ser102, Ser105, Ser108, Ser117, and, preferably Thr138, and most preferably Thr152, a recombinant osteopontin, e.g., a human or murine recombinant osteopontin, e.g., the osteopontin secreted from murine B3H cells, and a naturally occurring osteopontin, e.g., the naturally occurring human osteopontin secreted from human osteoblast cells (SEQ ID NO: 1). In a preferred embodiment threonine 152 is phosphorylated. In a more preferred embodiment, Ser26, Ser27, Ser81, Thr152 and Ser308 are phosphorylated.

As used herein, the term "active osteopontin peptide," refers to an osteopontin fragment that possesses at least one biological activity of a naturally occurring osteopontin. Preferred peptides include, but are not limited to, chemotactic peptides, e.g., peptides which comprise the amino acid sequence LVLDPK (SEQ ID NO: 2), or LVVDPK (SEQ ID NO: 3), or cell attachment peptides, e.g., peptides which comprise the amino acid sequence RGRDS (SEQ ID NO: 4). In preferred embodiments, the osteopontin peptides can be coated onto the material via covalent, non-covalent, or electrostatic interactions.

Alternatively, a chemotactic peptide can be a peptide which comprises an amino acid sequence X, X', D, Z, Z1, wherein X and X' are hydrophobic amino acids, D is aspartic acid, Z is proline (P), glycine (G), or serine (S), and Z' is a basic amino acid.

Preferred hydrophobic amino acids include asparagine (N), leucine (L), valine (V), isoleucine (I), glutamine (Q), or methionine (M). Preferred basic amino acid residues include lysine (K) and arginine (R). In one embodiment X and X' are selected from the group consisting of L, V, I, Q, M; Z is P, G, or S; and Z' is either K or R. In a most preferred embodiment X is L, X' is L, Z is G, and Z' is K.

Another preferred cell attachment peptide is GRGDS (SEQ ID NO: 5). GRGDS is a cell-binding domain which enhances cell attachment. A preferred cell-binding domain comprises the amino acid sequence VFTPVVPTVDTYDGRGDSVVYGLRSKSKKFRR (SEQ ID NO. 6).

As used herein, the phrase "in a releasable form," is intended to include osteopontin coated on top of the material in such a way that an osteopontin or a fragment thereof is capable of being released from the surface of the implant and performing its intended function *in vivo*, e.g., it is capable of establishing an osteopontin gradient in the proximity of an implant, preferably, within about 24 hours, more preferably within about 48 hours, of implantation. As used herein, "osteopontin gradient," refers to a protein gradient which results in the recruitment of cells, e.g., osteoblasts or osteoclasts, to an implant. Preferably, the osteopontin is non-covalently or electrostatically attached to the material. Non-covalent attachment is known in the art and includes, but is not limited to,

As used herein, the phrase "an osteopontin containing cell recruitment system" refers to a system in which osteopontin or a fragment thereof is introduced into a subject independent of an implant. Preferably, the osteopontin or a fragment thereof is introduced in the proximity of an implant in a form of a gel or a sponge. In other preferred embodiments, the osteopontin or a fragment thereof contained in a gel or a sponge is capable of generating a gradient of osteopontin in the proximity of an implant such that cells, e.g., osteoblasts or osteoclasts, are recruited to the implant. The phrase "an osteopontin containing cell recruitment system" is also intended to include chemotactic effects of osteopontin in facilitating wound healing and stimulating the recruitment of tissue remodeling cells from surrounding tissues. Tissue remodeling cells include mesenchymal, macrophage and granulocytes. Wound healing cells include, for example, cytokines which include TGFB and growth factors, cell-stimulating molecules and healing cells such as macrophages which help to clear chronic necrotic tissue from damaged tissue area.

The term "mesenchymal cell" is art recognized and is intended to include undifferentiated cells found in mesenchymal tissue, e.g., undifferentiated tissue composed of branching cells embedded in a fluid matrix which is responsible for the production of connective tissue, blood vessels, blood, lymphatic system and differentiates into various specialized connective tissues.

The term "growth factors" is art recognized and is intended to include, but is not limited to, one or more of platelet derived growth factors (PDGF), e.g., PDGF AA, PDGF BB; insulin-like growth factors (IGF), e.g., IGF-I, IGF-II; fibroblast growth factors (FGF), e.g., acidic FGF, basic FGF, β-endothelial cell growth factor, FGF 4, FGF 5, FGF 6, FGF 7, FGF 8, and FGF 9; transforming growth factors (TGF), e.g., TGF-β1, TGF-β1.2, TGF-β2, TGF-β3, TGF-β5; bone morphogenic proteins (BMP), e.g., BMP 1, BMP 2, BMP 3, BMP 4; vascular endothelial growth factors (VEGF), e.g., VEGF, placenta growth factor; epidermal growth factors (EGF), e.g., EGF, amphiregulin, betacellulin, heparin binding EGF; interleukins, e.g., IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14; colony stimulating factors (CSF), e.g., CSF-G, CSF-GM, CSF-M; nerve growth factor (NGF); stem cell factor; hepatocyte growth factor, and ciliary neurotrophic factor. Adams et al., "Regulation of Development and Differentiation by the Extracellular Matrix" *Development* Vol. 117, p. 1183-1198 (1993) (hereinafter "Adams et al.") and Kreis *et al.* editors of the book entitled "Guidebook to the Extracellular Matrix and Adhesion Proteins," Oxford University Press (1993) (hereinafter "Kreis et al.") describe extracellular matrix components that regulate differentiation and development. Further, Adams et al. disclose examples of association of growth factors with extracellular matrix proteins and

osteopontin is a recombinant osteopontin. In a most preferred embodiment, the site includes an implant as described herein.

The present invention also pertains to an osteopontin glue. The osteopontin glue includes osteopontin, a mucopolysaccharide and a multivalent metal. Suitable multivalent metals include copper, zinc, barium, calcium, magnesium, and manganese. The osteopontin glue can be administered to an area of tissue in need of repair, e.g., a wound, a cut, or other damaged tissue area, e.g., necrotic tissue. The osteopontin glue can be administered by methods known to those skilled in the art, such as, via injection. Administration of the osteopontin glue enhances tissue regeneration with concomitant removal of necrotic cells. In a preferred embodiment, the osteopontin glue can be used with an implant as described herein.

The osteopontin glues of the present invention may be given orally, parenterally, topically, or rectally. They are of course given by forms suitable for each administration route. For example, they are administered in tablets or capsule form, by injection, inhalation, eye lotion, ointment, suppository, etc. administration by injection, infusion or inhalation; topical by lotion or ointment; and rectal by suppositories. Injection or topical application is preferred.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion.

The osteopontin glues may be administered to humans and other animals for therapy by any suitable route of administration, including orally, nasally, as by, for example, a spray, rectally, intravaginally, parenterally, intracisternally and topically, as by powders, ointments or drops, including buccally and sublingually.

Regardless of the route of administration selected, the compositions of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of this invention may be varied so as to obtain an amount of the active ingredients which are effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient.

glycol; polyols such as glycerin, sorbitol, manitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; phosphate buffer solutions; and other non-toxic compatible substances employed in pharmaceutical formulations.

Wetting agents, emulsifiers and lubricants such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, stabilizers, preservatives or antioxidants can also be present in the compositions.

Methods of preparing these formulations or compositions include the step of bringing into association the osteopontin glue compositions of the present invention with a carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association the components of the osteopontin glue of the present invention with the carrier.

Liquid dosage forms suitable for administration of the osteopontin glue compositions of the invention include pharmaceutically acceptable emulsions and microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active ingredients, e.g. osteopontin, multivalent metals and mucopolysaccharides, the liquid dosage form can contain inert diluents commonly used in the art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propyleneglycol, 1,3-butyleneglycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethyleneglycols and fatty acid esters, sorbitan and mixtures thereof.

The osteopontin compositions can also contain adjutants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be insured by the inclusion of various anti-bacterial and anti-fungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, sugars, sodium chloride and the like into the compositions. In addition, prolonged absorption of the osteopontin compositions can be brought about by the inclusion of agents which allay absorption such as aluminum monosterate and gelatin e.g., collagen.

kinase may suppress the phosphorylation of a nearby residue, such as the mutually exclusive phosphorylation of hormone-sensitive lipase by cAMP-dependent protein kinase and calmodulin-dependent protein kinase.

Further modifications on the site and extent of phosphorylation can be achieved by expression of osteopontins with altered structures by differential splicing and post-translational modifications, as well as by the use of fragments and site-specific mutations at any one of these phosphorylation sites.

For phosphorylation by calcium/calmodulin kinase II, the reactions are carried out in the presence of 1.5 mM $CaCl_2$ and 3 µg calmodulin.

For phosphorylation by protein kinase C, the reactions are carried out in the presence of 8 µg/ml phosphatidylserine, 0.8 µg/ml of diacylglycerol, and 1 mM $CaCl_2$.

For autophosphorylation the reaction is carried out in the presence of 10 mM $MnCl_2$.

For phosphorylation by cGMP dependent protein kinase the reactions are carried out in the presence of 0.1 µM cGMP.

No additions are necessary for the phosphorylation of osteopontin by casein kinase I or mammary gland casein kinase.

Determination of phosphorylation sites in osteopontin:

After phosphorylation with ^{32}P-ATP and the desired kinase, osteopontin is digested with either trypsin, endopeptidase Glu-C, or endopeptidase Asp-N. The resulting peptides are separated by HPLC and the radiolabeled peptides sequenced. The position of the phosphorylated residue is determined by the coelution of radioactivity with the amino acid in that cycle.

Dephosphorylation of Osteopontin:

Osteopontin can be dephosphorylated by incubating the protein in either 100 µl 20 mM HEPES buffer, pH 8.5, and 1 unit of alkaline phosphatase, or 100 µl 20 mM acetate buffer pH, 5.0 and 1 unit of acid phosphatase, for several hours. Osteopontin can also be dephosphorylated by incubating the phosphoprotein with between 0.1 and 1 units of protein phosphatase 2A at 4°C for 1 h. Osteopontin can be also dephosphorylated by incubating the protein in 0.1 N NaOH for 1 h at 37°C.

O-glycosylation of osteopontin:

Osteopontin will be O-glycosylated by incubating the protein with commercially available rabbit reticulocyte lysate, which has been demonstrated by glycosylate nascent proteins in vitro (e.g., Starr, S.M. and Hanover, J.A. (1990) J. biol. chem. 265:6868-6873). Alternatively osteopontin could be O-glycosylated by using purified UDP-GalNAc:polypeptide N-acetylglactosaminyltransferase and UDP-N-acetylgalactosamine. The resulting O-glycosylated protein could be used to build more complex oligosaccharide side chains, using purified transferases and the appropriate sugar derivatives.

Glycation of osteopontin (nonenzymatic):

Nonenzymatic glycation involves the condensation of any sugar aldehyde or ketone, including phosphorylated derivatives of sugars, with either an α or ε amino group, resulting first in the rapid formation of a Schiff base. The Schiff base adduct can subsequently rearrange to the more stable Amadoriri product. For example, incubation of osteopontin with glucose, for several hours, will result in the formation β-pyranosyl Schiff base adduct, which will rearrange, with time, to the β-furanosyl Amadori product. Alternatively, the β-pyranosyl Schiff base adduct can be reduced at for 1 h at 22°C with 0.1% sodium horohydride to yield 1-deoxy-1-aminosorbitol derivative.

Sialation of osteopontin:

O-glycosylated osteopontin can be modified further by the addition of sialic acid. Briefly, 200 µg of osteopontin will be incubated with 0.5 milliunits of α 2,3-sialyltransferase in 100 µl 20 mM HEPES buffer pH, 6.5, containing varying concentrations of CMP-sialic acid for 1 h at 37°C. Whereas, N-glycosylated osteopontin can be sialated using α 2,6-sialytransferase and the conditions described above.

Deglycosylation of naturally occurring osteopontin:

Osteopontin, isolated from tissues, can be deglycosylated by the following methods:

Removal of N-linked oligosaccharides:

After treatment of osteopontin with neuranimidase to remove sialic acids, osteopontin is incubated overnight with 0.3 units of N-glycanase (Genzyme, Boston, MA) 100 µl of 20 mM HEPES buffer, pH 7.5, at 37°C.

Removal of O-linked oligosaccharides:

Asialoosteopontin is incubated for 1 to 6 h with 4 milliunits o-glycanase (Genzyme, Boston, MA) in 100 µl of 20 mM MOPS buffer, pH 6.0, at 37°C.

inorganic ion exchangers, Inorganic Ion Exchange Materials (ed. A. Clearfield) CRC Press, Boca Raton, FL, USA, pp 161-273, 1982). Titanium surfaces have a net negative charge at the pH values encountered in animal tissues, the pK being 4.0. This binding of cations is based on electrostatic interactions between titanium-linked 0- on the implant surface, and cations. The oxide layer is highly polar and attracts water and water-soluble molecules in general (Parsegian VA, J. Of Prosth Dent. 49(6):838-841, 1983).

2 The Bone-Titanium Layer

It is known that osscointegrated implants are characterized by the presence of an organic interfacial layer, containing no collagen fibrils, between the bone and the implant. This intervening layer in osseointegrated implants has been reported to stain with lanthanum and alcin blue and is both hyaluronidase and chondroitinase sensitive, suggesting proteoglycan content (Albrektsson T et al, Annals of Biomedical Engineering, 11, 1-27, 1983). The thickness of the glycan layer was found to vary with the biocompatibility of the implant material from 20 to 40 nm for Ti and 30 to 50 nm for zirconia (Albrektsson T, Jacobson M, J. Prosthet Dent 57:597-607, 1987). Establishment of this layer is reported to be critical for the success of the implant since it may provide an optimal interface between the dental implant and the newly formed bone (Nanci A et al, Cells and Materials, 4(1):1-30,1994).

Tissue response to commercially pure Titanium (cp Ti) was examined to characterize the bone implant interface. Lectin cytochemistry was used to detect glycoconjugates and immunocytochemistry for noncollagenous bone and plasma proteins. The composition of the titanium-matrix interface with that of natural bone interfaces such as cement lines and laminae limitantes was compared. The concentration of osteopontin (Opn) and alpha HS-glycoprotein at the bone titanium interface was consistent with the composition of cement lines at matrix-matrix interface and laminae limitantes at various cell-matrix interfaces. Furthermore, the data indicated that the interfacial layer between the bone and the implant is also rich in glycoconjugates containing sacharides such as galactose, a sugar residue found in relatively large proportion in osteopontin.

3 Bone Healing around Ti

The idea of osseointegration arose from studies of bone wound healing. Titanium chambers containing a transillumination system were inserted into the fibulae of rabbits to observe cellular changes during endosteal wound healing. At the completion of the study, retrieval of the titanium chambers required fracture of bone tissue that was integrated into the chamber surface. This incidental finding became the

5 Healing Of Bone Using Titanium Coated With Proteins

Recent studies have focused on improving the osseointegration of implants into bone by coating the Ti surfaces of implants with various substances including hydroxyapatite (Klein CP et al., Biomaterials. 15(2): 146-50, 1994; Jansen JA et al., Journal of Biomedical Materials Research. 25(8):973-89, 1991; Holmes RE, Plast. Reconstr Surg 63:626-636, 1979), fibronectin (Rutherford RB et al., International Journal of oral and Maxillofacial implants. 7(3):297-301,1992), and bone morphological proteins (BMP's) (Xiang W et al, Journal of Oral and Maxillofacial Surgery. 51(6):647-511, 993). Histological examinations of bone/titanium interface from such studies revealed various degrees of success in improving the osseointegration of Ti implants.

Titanium and Osteopontin

1 Protein Expression During Bone Formation

Morphological and histological studies on bone development categorize a linear sequence of cell differentiation progressing from an osteoprogenitor cell to preosteoblasts, osteoblasts and finally osteocytes and lining cells (Aubin JE et al., Analysis of osteoblast lineage and regulation of differentiation. In "Chemistry and Biology of Mineralized Tissue" (H. Slavkin and P Price, eds), pp 267-276. Excepta Medica, Amsterdam, 1992). Recently, the morphological and histological studies have been supplemented with the elucidation of some of the specific proteins secreted by bone cells at specific stages during their development. For example collagen type I is secreted by early and mature osteoblasts but decreases with late osteoblasts and osteocytes. Alkaline phosphatase is expressed by preosteoblasts and is accepted as a marker for osteoblasts. Osteopontin and bone sialoprotein are secreted by early osteoblasts, just prior to the onset of mineralization, but decreases as mineralization proceeds and osteoblasts mature and differentiate into osteocytes. Osteoblastic cells in vitro show an initial peak of Opn mRNA expression at early cultured times, followed by a second mayor peak of expression when the cultures begin to mineralize (Owen TA, J. Cell. Physiol. 143, 420-430, 1990; Strauss GP et al., J. Cell. Biol. 110,1368-1378, 1990). Osteocalcin is secreted by mature osteoblasts after the onset of mineralization. The order of appearance of proteins at bone interfaces, particularly with respect to type I collagen, is important in understanding the events leading to bone formation and turn over, and ultimately osseointegration.

the surface. This means that any cells which interact with the alloplast surface can only do so indirectly, through the absorbed protein layer.

The nature and amount of protein absorbed is specific to the alloplast composition (Uniyal S, Brash JL, Thromb. Haemost. 47, 285-290, 1982), depending on the physical and electromechanical properties of the given surface. It is conceivable that the absorbed protein contingent could determine what kind of cells interact with the alloplast surface (Bagambisa FB et al., Int. J. Oral Maxillof Implants 5, 217-226, 1994). Cell contact with the substrate is maintained by the formation of subcellular spatially and morphologically defined adhesion sites called focal adhesions. Focal adhesion are within 15 to 30 nm proximity of the substrate (Izzard CS, Lochner RL, J. Cell Sci. 21:129-159, 1976) and are about 2 to 10 μm long and 150 to 500 nm wide (Burridge K et al, Ann. Rev. Cell Biol., 487-525, 1988). Although the different phenomenological response of cells to material surfaces has been attributed to wetability, this can only be a first approximation (Parsegian VA, J. Of Prosth Dent. 49(6):838-841, 1983). It appears more useful to talk about the ability of the surfaces to interact with the key molecules involved in the orchestration of the post implantation interfacial events. If a material surface can not bind the macromolecules supportive of osteoblast function, the material is not likely to make a good bone implant. One way of getting bone cells to appose bone tissue onto the implant surface might be through having or creating surfaces that are attractant to the macromolecules responsible for events like cell phenomenology, growth and differentiation (Bagambisa FB et al. Int. J. Oral Maxillof. Implants 5:217-226, 1994).

The absorption onto Ti of aqueous solutions of matrix or matrix-like proteins has resulted in significant increases in the number of cells bound. This effect has been reported (Burridge K et al. Ann. Rev. Cell Biol. 487-525, 1988) and indicates that a specific cell receptormatrix protein interaction is a more efficient means of attachment than the undefined process of cell-Ti interaction.

Histological information is available on the interface between bone and implant material, but the understanding of the mechanisms operating when an implant is inserted into bone is limited and the concepts are speculative.

The process of integration is going on in an aqueous environment. When two bodies make contact, it is because they prefer each other to the intervening water or whatever else is originally between them. In the vicinity of an electrical charge, a molecule will turn to keep its attractive end close to the intruding charged body (Parsegian VA, J. Of Prosth Dent. 49(6):838-841, 1983). Small amounts of positively charged calcium ion will bind to certain electrically negative surface groups, displacing the water and replacing it with a bridge of (-) (+), (+) (-) configurations between

columns, Biochim. Biophys. Acta. 160, Pp 301-310, 1968). Glycosaminoglycans interact electrostatically with hydroxyapatite surface (Embery G and Rolia G, Interaction between sulphated macromolecules and hydroxyapatite studied by infrared spectroscopy. Acta Odontol. Scand, 38, 105-108, 1980). It has been shown that calcium absorbs to the surfaces after treatment with $CaCl_2$. The absorption of calcium onto the titanium implant surface when exposed to body fluids, increase its biocompatibility with bone and induce a subsequent adsorption of calcium binding macromolecules on to the implant surface. The surface characteristics of TiO_2 probably change from an anionic to a cationic state by the adsorption of calcium to the surface which will be subsequently have an increased ability to absorb acidic macromolecules like Opn. The results of the study were consistent with the proposal that calcium binding is a major mechanism by which proteins adsorb to TiO_2.

The present invention is further illustrated by the following Examples which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, and published patent applications) cited throughout this application are hereby expressly incorporated by reference.

Examples

Titanium, plastic, glass and chromocobalt (CrCo) surfaces were coated with human recombinant OPN. Attachment and proliferation of human osteoblasts by means of matrix formation markers was evaluated using uncoated surfaces as a control. Also the amount of adhesion protein that can be coated to these surface was investigated.

The human recombinant phosphorylated form of osteopontin (rhOpn) was used as an adhesion molecule. This form of osteopontin migrates on 10% SDS-gels with an apparent molecular weight of 78Kd, making it easy to differentiate from osteopontin secreted by osteoblasts which migrates in the same gels with an apparent molecular weight of 58Kd.

The experiments outlined below investigate the expression and mineralization of extra cellular matrix components in human osteoblasts cultured on titanium disks, plastic, glass and chromocobalt surfaces coated with recombinant osteopontin. The adhesion molecule rhOPN used as a coating for these surfaces enhances attachment and proliferation of human osteoblasts cell lines, and increases the expression of matrix components when compared against uncoated surfaces.

N NaOH. ^{3}H-thymidine incorporation into TCA insoluble material was used as an index of cell proliferation. The material collected was mixed with scintillation liquid (Begman). The amount of radiation generated was compared between cells grown in titanium disks uncoated, and titanium disks coated with OPN.

Synthesis of osteopontin (Opn) and bone sialoprotein (BSP), and their secretion and deposition into the extracellular matrix.

Osteopontin and BSP were extracted from the extracellular matrix of HOS cells cultured on Ti disks or Ti disks coated with the recombinant Opn with lysis buffer (20 mM phosphate buffer, pH, 7.2, containing 150 mM NaCl, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 5mM benzamidine, 0.1 mM e-amino caproic acid, 0.1 b-hydroxy mercuribenzoate, 0.1 mM pyrophosphate, 1mM sodium fluoride, 1mM sodium orthovanadate and 10 mM EDTA). Samples were then processed for Gel electrophoresis.

Western blot analysis: Cell layer proteins and conditioned media was electrophoresed in 10% SDS-polyacrylamide slab gels at 150 volts for 4h. For Western blot analysis resolved proteins in gels were transferred by semi-dry blotting onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH), gel transfers were carried out for 90 min. at 12 V in 0.025 M Tris-glycine buffer, pH 8.2, containing 20% methanol and 0.01% Tween 20 and 10% nonfat dry milk, then incubated with rabbit anti-mouse osteopontin (Ashkar S, et al., New York Academy of Science 760:296-298, 1995) in 20 mM Phosphate buffer, pH 7.4, containing 150 mM NaCL, 0.1% Tween 20 and 1% nonfat dry milk. After 1h, the membranes were washed 3 times with 20mM Phosphate buffer, pH 7.4, containing 150 mM NaCl, 0.1 % Tween 20, then incubated with horseradish peroxidase-conjugated goat anti-rabbit 1g antibodies for 1h. Following several washing steps, the membranes were developed with ECL. Nonspecific interaction was assessed by the interaction of the primary and secondary antibodies with rabbit serum albumin.

Identification of proteins was made running the samples collected in a 7.5% SDS-polyacrylamide slab gels at 150 volts for 4h. Then, the gels were stained by immersion in Coomassie blue for 24 hours. The gel was washed with 10% Acetic Acid, 20% Methanol, 70% ddWater, and the proteins identified by molecular weight against the standards ran with the samples.

were lysed with 10% TCA and solubilized in 5 ml scintillation fluid. All measurements were done in triplicates and graphed as mean ± Standard error of the mean.

The initial events following seeding of cells onto Ti surfaces include the attachment, migration and proliferation of the seeded cells. Coating Ti disks with 50 µg of rhOPN enhanced by 1100% the attachment of HOS cells to Ti disks (Figure 2), after 30 min. These results are consistent with the role of osteopontin in promoting cell attachment and spreading.

Example 3: Proliferation of HOS cells on Ti surfaces coated with phosphorylated human recombinant Opn.

Cell proliferation was determined by the rate of ^{3}H-Thymidine incorporation into DNA. Cells labeled with ^{3}H-Thymidine were seeded for 6 hours, then lysed with TCA. The TCA insoluble material was collected and resuspended in 0.5 N NaOH. ^{3}H-thymidine incorporation into TCA insoluble material was used as an index for cell proliferation. Rate of proliferation is expressed as cpm/1000 cells/6h. Control group: 254,54, rhOPN group: 560,83. All measurements were done in triplicates and reported as mean ± Standard error for the mean.

Since rhOPN promoted cell attachment to Ti disks, it was of interest to examine whether the protein had any effect on the proliferation of HOS grown on Ti disks. Measurement of the rate of proliferation of HOS cells grown on coated or uncoated Ti disks showed that the proliferation rate of cells grown on rhOPN coated Ti disks was approximately twice (Figure 3) the proliferation rate of cells grown on uncoated Ti disks.

Example 4: Secretion of osteopontin and BSP by HOS cells growing on coated Ti disks.

Cell layer proteins and conditioned media was electrophoresed in 10% SDS-polyacrylamide slab gels at 150 volts for 4h. The resolved proteins were transferred by semi-dry blotting onto nitrocellulose membranes for 90 min. at 12 V in Transfer Buffer. Then, the membranes were incubated with either rabbit anti-mouse osteopontin or rabbit anti-mouse BSP. After 1 h, the membranes were washed 3 times with PBST. Then incubated with horseradish peroxidase-conjugated goat anti-rabbit Ig antibodies for 1h. Following several washing steps in PBST, the membranes were developed with ECL as described by the manufacturer (Amersham, London).

Osteopontin and BSP were extracted from the extracellular matrix of HOS cells cultured on Ti disks or Ti disks coated with the recombinant Opn with lysis buffer. Samples were then processed for Gel electrophoresis. Western blot analysis for OPN

disks HOS cells attach, proliferate and differentiate at a slower rate than when cultured on coated disks. Furthermore, HOS cultured on coated disks synthesize an extracellular matrix that mineralizes within two weeks. In several respects HOS cells grown on Ti surfaces coated with rhOPN develop in a manner similar to cells grown on plastic dishes.

Example 7: Attachment of HOS cells to surfaces coated with OPN

500 cells were plated on coated plastic, glass or chromocobalt surfaces and incubated at 37°C in a humidified atmosphere (95% air 5% $C0_2$). Surfaces were coated with either human recombinant phosphorylated OPN (rhOPN) or unphosphorylated OPN. Fibronectin coated surfaces were used as a control. After 1 hour, unattached cells were removed and the surfaces were washed with PBS. The total number of attached cells was determined for the total cpm released for the surfaces after the cells were lysed with 10% TCA and solubilized in 5 ml scintillation fluid. All measurements were done in triplicates. The results are outlined in Table 2 below.

TABLE 2

Surface	**% total attached**
plastic	
OPN	43.6
OPN-p	90.8
fibronectin	91.6
glass	
OPN	37.2
OPN-p	98.1
fibronectin	89.6
chromocobalt (CrCo)	
OPN	4
OPN-p	69.2
fibronectin	54.8

OPN = unphosphorylated OPN

OPN-p = phosphorylated OPN

Example 9: *In Vivo* Studies of Ti coated rhOPN implants

Forty implants (5 per quadrant) were placed in four Haundel/Labrador dogs after extraction of four premolars (PM1-PM4) and one molar (M1), and a three month healing period. Eight hollow screw Ti implants were coated with rhOPN. Eight uncoated implants served as controls. The remaining implants were coated with 3 additional different molecules denoted as study 2, study 3, and study 4.

Prior to implant placement, core samples from the donor place were taken to histologically analyze bone quality after extractions. This procedure, also ensured a hollow space for bone ingrowth inside the coated and uncoated implants. Dogs were sacrificed after 4 and 8 weeks.

Implants were recovered for histological analysis. Each implant was sectioned vertically. The core inside the hallow implant was removed using liquid nitrogen. Decalcified sections were embedded in paraffin and stained using Herovichi's techniques to differentiate immature from mature collagen. Light microscopy at 4X and 40X magnifications were used to compare histological differences between rhOPN coated implants and uncoated implants.

The *in vivo* results show enhanced bone healing around coated implants. Uncoated implants show normal bone healing characterized by granulation tissue and a few areas of vascularization and matrix deposition after four weeks. These results demonstrate that coating titanium implants with rhOPN reduces healing time around dental implants.

The results outlined above demonstrate that coating of different surfaces, e.g., titanium disks, glass, plastic, or CrCo, with phosphorylated human recombinant osteopontin enhances the rate of attachment and proliferation of human osteoblast cell lines *in vitro* when compared to uncoated surfaces. This enhancement is demonstrated by better attachment and proliferation of the cells, increased production of the extracellular matrix components, and its faster calcification. These results also contribute to the understanding of the molecular events that may be occurring in the healing of bone around the implants.

Equivalents

Those skilled in the art will be able to recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the following claims.

12. The implant of claim 1 wherein the osteopontin further possesses at least one osteopontin polypeptide possessing chemotactic activity.

13. The implant of claim 12 wherein the chemotactic polypeptide comprises the amino acid sequence (SEQ ID NO: 2) or (SEQ ID NO: 3).

14. An implant of claim 12 wherein the chemotactic polypeptide comprises the amino acid sequence:

XX'DZZ'

wherein X and X' are hydrophobic amino acids;
D is Aspartic Acid;
Z is Proline, Glycine, or Serine;
Z' is a basic amino acid.

15. An implant of claim 14 wherein X and X' are selected from the group consisting of Leucine, Valine, Isoleucine, Glutamine, and Methionine; Z is selected from the group consisting of Proline, Glycine and Serine; and Z is selected from the group consisting of Lysine and Arginine.

16. An implant of claim 15 wherein X is Leucine, X' is Leucine, Z is Glycine, and Z' is Lysine.

17. The implant of claim 1 wherein the osteopontin further comprises at least one osteopontin polypeptide possessing cell attachment activity.

18. The implant of claim 17 wherein the cell attachment peptide comprises the amino acid sequence (SEQ ID NO: 4) or (SEQ ID NO: 5).

19. An implant of claim 18 wherein the cell attachment peptide comprises (SEQ ID NO. 6).

20. An osteopontin containing titanium implant, comprising:
a releasable form of phosphorylated osteopontin or an active fragment thereof in combination with titanium suitable for use *in vivo* within a subject forming an osteopontin containing titanium implant.

21. The implant of claim 20 wherein the titanium implant is a dental implant.

28. A method for inducing new tissue formation in a subject at a site where tissue formation is needed comprising:

adding osteopontin to a subject at a site where tissue formation is needed, wherein the osteopontin induces new tissue formation about the site.

29. The method of claim 80 wherein the osteopontin is a recombinant osteopontin.

30. An osteopontin glue, comprising osteopontin, a mucopolysaccharide and a multivalent metal.

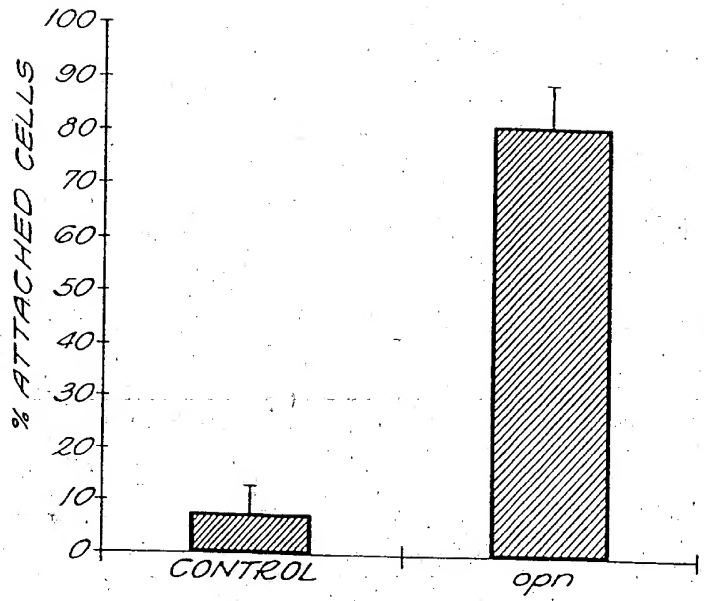

FIG. 2

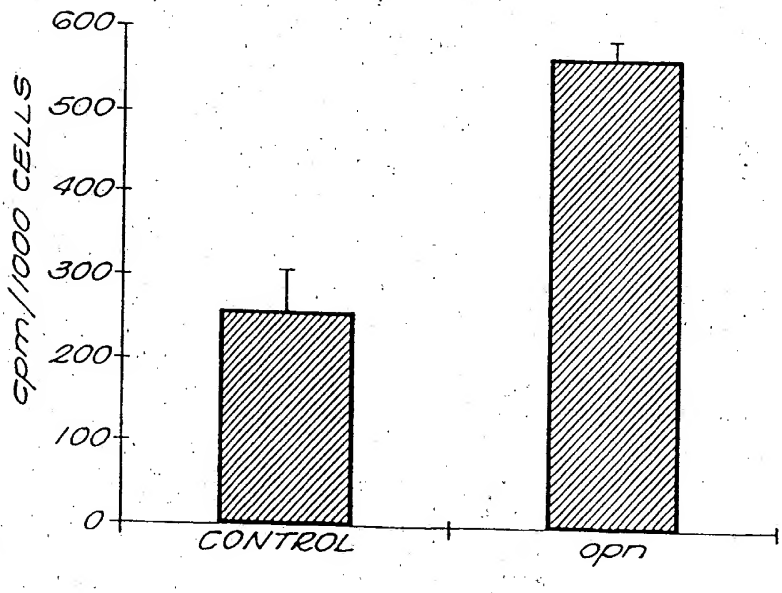

FIG. 3

SEQUENCE LISTING

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Inte. ..tional Application No
PCT/US 98/16888

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 93 07910 A (ISIS INNOVATION) 29 April 1993 see claims; examples 1-3 & US 5 508 267 A cited in the application	1-30
A	DIJK VAN S ET AL: "EVIDENCE THAT A NON-RGD DOMAIN IN RAT OSTEOPONTIN IS INVOLVED IN CELL ATTACHMENT" JOURNAL OF BONE AND MINERAL RESEARCH, vol. 8, no. 12, December 1993, pages 1499-1506, XP002047131 see abstract	1-19
A	NASU K ET AL: "EXPRESSION OF WILD-TYPE AND MUTATED RABBIT OSTEOPONTIN IN ESCHERICHIA COLI, AND THEIR EFFECTS ON ADHESION AND MIGRATION OF P388D1 CELLS" BIOCHEMICAL JOURNAL, vol. 307, no. 1, 1 April 1995, pages 257-265, XP002047130 see page 264	1-19
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1

INTERNATIONAL SEARCH REPORT

Information on patent family members

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